Fluorescence Properties of Octopine Dehydrogenase†

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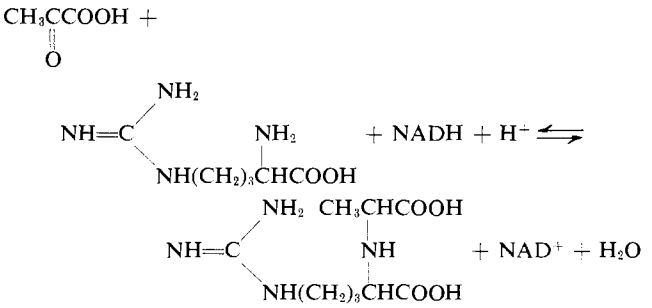
ABSTRACT: The fluorescence properties of the monomeric octopine dehydrogenase and its binary and ternary complexes are investigated under a variety of conditions. The intrinsic fluorescence of the protein is due to tryptophan, the contribution of tyrosine being absent or negligible. The fluorescence quantum yield (60% with respect to tryptophan) decreases by a factor around 2 in the temperature range 3–40° and is insensitive to changes in ionic strength and pH (in the range 5–9). Extreme pH values produce a substantial quenching accompanied by a red shift (from 330 to 340 nm). There is an enhancement of the NADH fluorescence intensity in the binary and ternary complexes, with a parallel shift of the fluorescence maximum toward the blue. The fluorescence of the protein is quenched upon binding of both NAD^- and NADH. The binding curves obtained by following the fluorescence quenching, contrary to the case of some protomeric dehydrogenases, exactly parallel those obtained by following the enhancement of coenzyme fluorescence. At room temperature and in the pH range 6–9, the dissociation constant is 20 μM for NADH and 250 μM for NAD^+. The affinity of NADH to the enzyme is greatly increased in the ternary complex containing the substrates arginine (K_d = 2.5 μM) and octopine (K_d = 0.9 μM). The tight binding in the ternary complex (octopine dehydrogenase–NADH–octopine) permits an accurate titration of the enzyme active-site concentration. The comparison of the various dissociation constants permits a preliminary estimate of the order of ligand release from the enzyme ternary complexes.

Octopine dehydrogenase catalyzes the interconversion between octopine and pyruvate plus arginine in the presence of nicotinamide adenine diphosphonucleotide (NAD–NADH).



The enzyme is a monomer (Olomucki *et al.*, 1972), which is of particular interest considering that most dehydrogenases so far investigated are dimers, tetramers, or higher oligomers. The enzyme has been investigated by several approaches (Thoai *et al.*, 1969; Pho *et al.*, 1970; Oriol and Olomucki, 1972); recently, its amino acid composition has been determined (Olomucki *et al.*, 1972).

No fluorescence investigation has been carried out so far on this enzyme. On the other hand, fluorescence techniques have been shown to be useful for obtaining information on structural properties of the active site of dehydrogenases (see, for instance, Velick, 1958, Weber, 1968, and Brand and Witholt, 1967) to determine enzyme–nucleotide binding constants (see, for instance, Price and Radda, 1971, Su and Wilson, 1971, and Theorell and Yonetani, 1964), and for titrating the enzyme active sites (Taniguchi *et al.*, 1967; Luisi and Favilla, 1970; Dickinson, 1970). These considerations, combined with an interest in comparing the enzyme–coenzyme interaction between the monomeric enzyme and the other dehydrogenases, prompted the present research.

Material and Methods

Enzyme. Octopine dehydrogenase has been prepared and assayed as previously described (Thoai *et al.*, 1969). A molar extinction coefficient of 4.33×10^4 M^{-1} cm^{-1} at 280 nm has been used for the calculation of the enzyme concentration. The activity of the enzyme preparations used in this work ranged from 90 to 100%; the ratio of the 280 nm/260 nm absorbance ranged between 1.92 and 2.00.

Coenzymes. NADH has been prepared starting from commercial samples (Boehringer, purity degree II) according to the procedure described by Holbrook and Wolfe (1972). Only the fractions having an optical absorbance ratio at 340 nm/260 nm of 0.45 were used for the binding studies. The concentrated NADH solution was diluted to the desired concentration value with buffer. NAD has been prepared starting from commercial samples (Boehringer, purity degree II) according to a partial modification of the procedure described by Holbrook and Wolfe (1972). A volume of 300 ml of 0 to 0.3 M LiCl gradient instead of 15 mM HCl was used for the elution. From about 150 mg of starting material approximately 10 ml of concentrated NAD solution, having an optical absorbance ratio at 280 nm/260 nm of 0.22, was obtained, and only this fraction was used for the binding studies.

The NADH concentration was determined by absorption measurement at 340 nm and NAD^+ concentration by absorption measurement at 260 nm, assuming ϵ_{340} and ϵ_{260} to be 6.22×10^3 and 18×10^3 for NADH and NAD^-, respectively. The coenzyme solutions were prepared each time immediately before use. For this particular enzyme, high-purity commercial samples of the two coenzymes (Boehringer, purity degree I, Sigma, grade V) gave the same result. NAD prepared according to Dolin and Jacobson (1963) also gave the same percent quenching and the same binding constants.

Particular care was taken in order to avoid fluorescent im-

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purities in the NAD^+ preparations (see, for instance, Dolin and Jacobson, 1964), or the formation of NADH *in loco* during the titration with NAD^+. The possible presence of coenzyme fluorescence was investigated at the end of a quenching experiment by scanning the excitation light between 300 and 350 nm *ca.* at the highest emission sensibility of the fluorometer. No fluorescence emission was detected under conditions at which 1 μM of NADH gave 30% of the full-scale response. Octopine has been obtained as previously described (Pho *et al.*, 1970). L-Arginine was purchased from Fluka.

Fluorescence Measurements. The instrumentation and technique for the fluorescence measurements have already been described (Luisi and Favilla, 1970). The use of small cells (0.5 cm or less) allowed relatively high coenzyme concentrations to be used without the problem of a too high optical density. Typically, the total optical density (sum of the absorptions of enzyme and coenzyme at the wavelength of emission and excitation) was kept below 0.3. In the experiments illustrated in Figure 3, for instance, the maximal optical density is 0.19 in the case of NADH and 0.23 in the case of NAD^+. Corrections for inner filter effects were carried out according to Parker (1968). The value of d_1 and d_2 (the two geometrical parameters used by Parker) have been obtained through a calibration between fluorescence and concentration of solutions of protein in buffer. The correction factor used in these experiments was, for instance, 1.1 and 1.3 for the first and last point, respectively, of the NAD^+ binding in Figure 3.

Treatment of the Data. The basic assumption for the elaboration of the data is that the per cent of the fluorescence change is directly proportional to the enzyme–coenzyme complex, so that the molar fraction ν of the enzyme bound at each concentration of coenzyme is given by: $\nu = [ES]/[E]_{tot} = \Delta F/\Delta F_{max}$, where the enzyme concentration is expressed in normality (*i.e.*, active-site concentration). ΔF_{max}, the maximal fluorescence change, corresponds to complete saturation by the ligands and ΔF is the actual fluorescence change, obtained at a given concentration of ligand.

The accuracy by which ΔF_{max} is known is critical for the correct evaluation of the binding constants. In our case, ΔF_{max} can be evaluated with considerable accuracy (see Figure 3B) as the double-reciprocal plots are linear (at least after the first 10–20% of the titration range, not reported in Figure 3B). This linearity is a general feature for monomeric enzymes when the dissociation constant is larger than the enzyme concentration, so that $[NADH]_{free} \simeq [NADH]_{tot}$.

Comparison of Figure 3A,B shows the importance of the double-reciprocal plots for determining ΔF_{max}. The qualitative visual inspection of Figure 3A would probably give an apparent ΔF_{max} which is appreciably lower than that obtained from Figure 3B. A lower ΔF_{max} would give a much smaller dissociation constant, and distortions in the corresponding Hill and Scatchard plots. Actually, a good check of the accuracy of the chosen ΔF_{max} is whether one obtains a good extrapolation to $\nu = 1$ in the Scatchard plots (the accuracy to which $[E]_{tot}$ is known does not effect this extrapolation in the case of a loose binding). When the dissociation constant is small, so as to be numerically comparable to the enzyme active-site concentration used in the actual experiments, the double-reciprocal plots in terms of $[NADH]_{tot}$ are no longer as linear as in Figure 3B (they would be linear if $[NADH]_{free}$ were used in the plot and in the case of a simple binding). ΔF_{max} is obtained in this case by extrapolation of the very last part (saturation larger than 75–80% of the enzyme sites) of the double-reciprocal plot. The extrapolation of ΔF_{max} from the first part of the double-reciprocal plot would yield too high a value, which in turn would give too low a value for the number of binding sites in the Scatchard plots. If the enzyme active-site concentration is known with accuracy by some other independent method, the correct value of ΔF_{max} should produce a Scatchard plot which extrapolates to $\nu = 1$ (or in general to $\nu = n$, if the enzyme molar concentration is used instead of the enzyme normality).

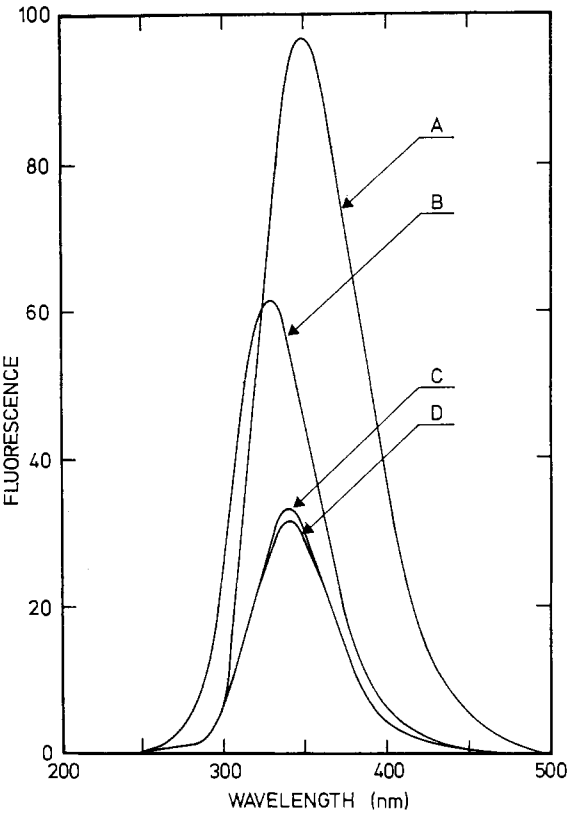


FIGURE 1: Emission spectra of octopine dehydrogenase at different pH's: (A) L-tryptophan in water or 0.1 M sodium phosphate buffer (pH 7.0); (B) octopine dehydrogenase, pH 4, 5, 6, 7, 8, 9 (0.1 M sodium phosphate or glycine buffer); (C) octopine dehydrogenase, pH 2.0 (0.1 M glycine–HCl buffer); (D) octopine dehydrogenase, pH 12.0 (0.1 M glycine–NaOH buffer). All solutions have the same optical density (0.20) at the excitation wavelength (280 nm); temperature 23 ± 1°. The spectra are uncorrected.

Results

Emission Spectra of Octopine Dehydrogenase. Figure 1 reports the emission spectra of octopine dehydrogenase under different conditions, as well as of tryptophan in water, for an excitation light of 280 nm. The shape of the emission spectrum of the native enzyme does not change by changing the excitation wavelength. This can be taken as an indication that the fluorescence of the enzyme is mostly due to tryptophan residues, the contribution of tyrosine being absent or negligible (Eisinger, 1969). The lack of tyrosine contribution to the overall fluorescence of proteins, is a well-documented phenomenon (Teale, 1960; Cowgill, 1969). The wavelength of the emission maximum of the native enzyme is at 330 nm, just as in many other dehydrogenases, which corresponds to a 20- to 30-nm blue shift with respect to simple peptides containing tryptophan. As well known, this can be taken as an indication of a hydrophobicity around the emitting tryptophans of the protein. From the comparison of the areas of the emission spectra of tryptophan in water and 100% active octopine dehydrogenase one can calculate that the quantum yield of octopine de-

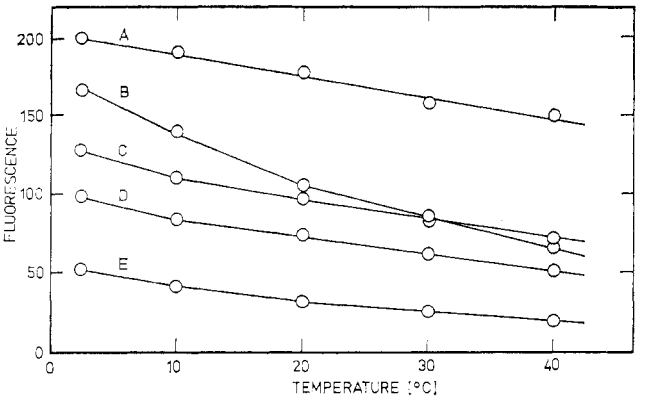


FIGURE 2: Dependence of the fluorescence intensity on the temperature: (A) horse liver alcohol dehydrogenase; (B) L-tryptophan; (C) L-tryptophylglycine; (D) octopine dehydrogenase; (E) glycyl-L-tryptophan. All solutions have the same optical density (0.20) at the excitation wavelength (280 nm); emission 330 nm; temperature 23 ± 1°; 0.1 M sodium phosphate buffer (pH 7.0).

hydrogenase (uncorrected for the tyrosine and phenylalanine absorption) relative to tryptophan is 60% at room temperature. This quantum yield is considerably lower than that of horse liver alcohol dehydrogenase (*ca.* 150%) or lactate dehydrogenase (150–300%, McKay and Kaplan, 1964) and is of the same order of magnitude, for instance, as that of yeast alcohol dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase from various sources.

There is no influence of pH on the emission properties of octopine dehydrogenase in the pH range 4–9. This seems to suggest that the hydrophobicity of the tryptophan residues is not disturbed by the "external" concentration of H^+. More drastic pH conditions cause a red shift and a consequent remarkable decrease in quantum efficiency (see Figure 1). A similar change is produced by urea treatment.

The fluorescence intensity is proportional to the enzyme activity. The decrease of the quantum yield is not accompanied by any red shift of the fluoresence maximum (at least up to an activity of 40–50%).

The influence of temperature on the fluorescence intensity is shown in Figure 2. The temperature coefficient of the fluorescence intensity of octopine dehydrogenase parallels that of horse liver alcohol dehydrogenase, and that of simple dipeptides containg tryptophan. There is no shift of the fluorescence maximum in the investigated temperature range (2–40°).

There is no influence of the ionic strength on the emission properties of octopine dehydrogenase, at least in phosphate buffer (pH 7.0) for the range of 0.5–0.02 M.

Effect of the Coenzyme Binding on the Enzyme Fluorescence. Addition of NADH or NAD^+ to the enzyme produces a quenching of the tryptophan fluorescence. This is shown in Figure 4 in typical experiments at pH 7.0 phosphate buffer. In the NAD quenching experiments particular care has been given to check the coenzyme purity (see Experimental Section). The maximal quenching caused by the coenzymes is evaluated through the double-reciprocal plots. As shown in Figure 3B, it corresponds to 60 and 35% for NADH and NAD^+, respectively. These ΔF_{max} values are independent of temperature (in the range 2–40°) and pH (range 6–9) within 10%. There is no shift of the wavelength of the fluorescence maximum upon coenzyme binding.

The fluorescence of NADH is enhanced in the binary octopine dehydrogenase–NADH complexes, and even more so in the ternary octopine dehydrogenase–NADH–arginine and

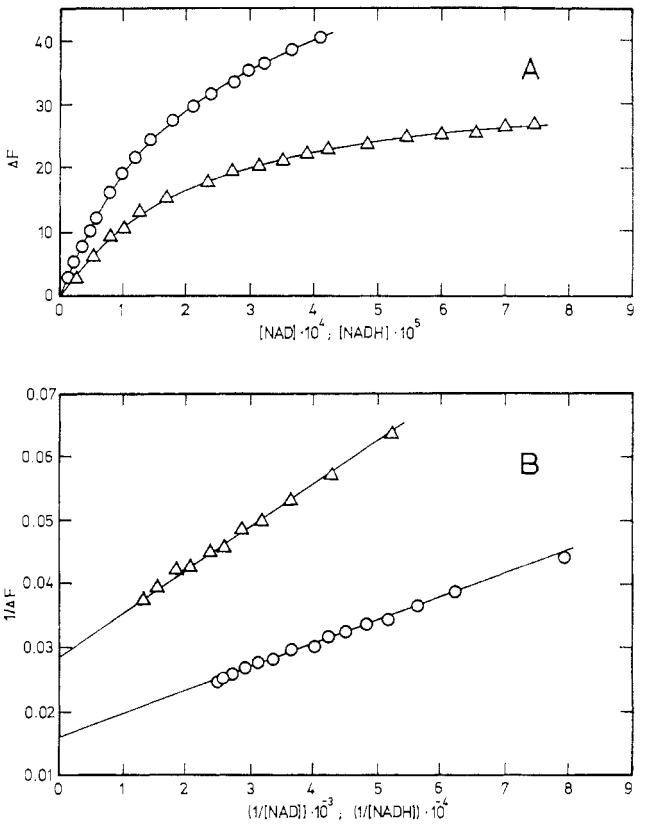


FIGURE 3: Fluorescence quenching in octopine dehydrogenase: (Δ) data relative to NAD^+ binding; (○) data relative to NADH binding. Conditions were 4.6 μM enzyme for the NADH quenching and 6.0 μM enzyme for the NAD^+ quenching; pH 7.0, sodium phosphate buffer (0.1 M); temperature 20 ± 1°; excitation 290 nm, emission 330 nm: (A) direct plot; (B) double-reciprocal plot.

octopine dehydrogenase–NADH–octopine complexes. The wavelength of the fluorescence maximum is shifted toward the blue. Figure 4 reports the NADH fluorescence of binary and ternary complexes, for a constant concentration of enzyme and NADH. For the quantitative evaluation of the enhancement of the NADH fluorescence in the different cases, the relative amount of NADH free and bound should be taken into account, as the fluorescence enhancement is only due to the bound coenzyme. For instance in the binary octopine dehydrogenase–NADH complexes only 16% of the NADH is bound for the conditions of Figure 4 (see binding constants in the next section). On this basis one obtains for NADH a fluorescence enhancement by a factor 6.5 in the binary complex, by a factor 10 in the ternary complexes with arginine and by a factor 14 in the ternary complexes with octopine. The fluorescence of NADH is not affected by arginine or octopine in the absence of the enzyme, nor is the enzyme tryptophan fluorescence affected by arginine or octopine in the absence of coenzyme. There is no effect of pyruvate on the NADH fluorescence or the protein fluorescence.

The interaction between NADH and octopine dehydrogenase results in energy transfer between the emission of the protein and the absorption of the coenzyme. In the excitation spectrum, obtained for an emission wavelength of 450 nm, is in fact present a sizable band corresponding to the protein absorption.

Both the enhancement of fluorescence and the shift of the fluorescence maximum when NADH is bound in binary or ternary complexes are rather common features in dehydroge-

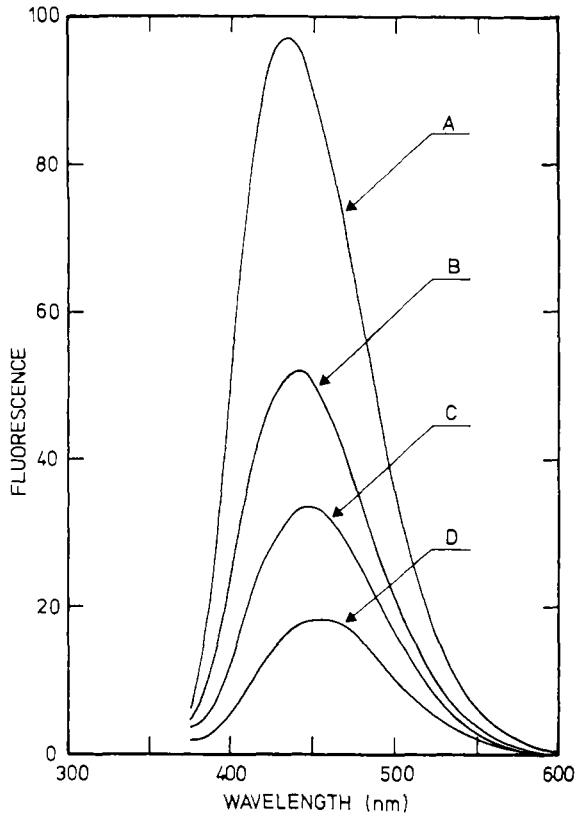


FIGURE 4: Enhancement of the NADH fluorescence in binary and ternary complexes of octopine dehydrogenase: (A) octopine dehydrogenase-NADH-octopine; (B) octopine dehydrogenase-NADH-L-arginine; (C) octopine dehydrogenase-NADH; (D) NADH alone (reference). Conditions were 6.46 μM octopine dehydrogenase, 16 μM NADH, 0.02 M octopine, and 0.02 M arginine. Sodium phosphate buffer 0.1 M, pH 7.0; temperature 20 ± 1°; excitation 340 nm. The spectra are uncorrected.

nases (see, for instance, Velick, 1961, Boyer and Theorell, 1956, Winer *et al.*, 1959, and Dickinson, 1970).

Determination of the Enzyme-Coenzyme Binding Constants. The quantitative evaluation of the quenching process permits the determination of the binding constants for NAD^+ and NADH. The method has been previously discussed (Luisi and Favilla, 1970; see also Experimental Section).

Figure 5 reports Hill plots of the binding of coenzyme to octopine dehydrogenase. As expected in the case of a monomeric enzyme, the binding is simple, with $n = 1$. The affinity of NADH ($K_d = 20\ \mu M$) is one order of magnitude higher than that of NAD^+ ($K_d = 250\ \mu M$). This feature is characteristic of several dehydrogenases. The binding of NADH to octopine dehydrogenase has been also measured by gel filtration techniques (Olomucki *et al.*, 1972). The value of the $K_d = 20\ \mu M$ agrees well with the value determined by quenching of the tryptophan fluorescence. There is no variation of K_d of NADH in the pH range 6-9, whereas K_d of NAD^+ increases slightly with increasing pH. K_d for both NAD^+ and NADH are insensitive to changes in the buffer ionic strength in the range 0.02-0.5 M. In Table I, some miscellaneous data are reported, together with the actual ΔF_{max} values of each experiment.

Figure 5 also reports the Hill plot of the binding of NADH to the enzyme saturated by arginine. The binding is also simple with $n = 1$, but the affinity of the coenzyme is increased by an order of magnitude in the ternary complex ($K_d = 2.5\ \mu M$ at pH 7.0 phosphate buffer and room temperature) with respect to the binary octopine dehydrogenase-NADH complex. It

TABLE I: Binding Constants of Octopine Dehydrogenase to NAD^+ and NADH[a] Determined by Quenching of the Tryptophan Fluorescence.[b]

pH/Buffer:	Coenzyme	Second Ligand	K_{diss} (μM)	Δ-F_{max}
7.0/0.1 M Pho[c]	NADH		20	62
7.0/0.1 M Pho[c]	NAD		260	33
7.0/0.1 M Pho[c]	NADH	0.02 M Arginine	2.5	53
7.0/0.1 M Pho[c]	NAD	0.02 M Arginine	180	40
7.0/0.1 M Pho[c]	NADH	0.02 M Octopine	0.9	52
7.0/0.1 M Pho[c]	NADH	0.1 M Pyruvate	18	60
7.0/0.1 M Pho[c]	NADH	0.1 M Arginine	2.4	58
6.0/0.05 M Pho-Gly[d]	NADH		16	63
6.0/0.05 M Pho-Gly[d]	NAD		230	33
8.0/0.05 M Pho-Gly	NADH		22	60
8.0/0.05 M Pho-Gly	NAD		350	37
9.0/0.05 M Pho-Gly	NADH		25	69
9.0/0.05 M Pho-Gly	NAD		350	35

[a] Enzyme concentration between 4.6 and 6.5 μM; temperature 22 ± 1°. [b] A mixture of phosphate and glycine was used in order to have the same buffer in the range of pH investigated. [c] Pho = sodium phosphate buffer. [d] Gly = glycine-HCl or glycine-NaOH buffer.

is interesting to remark that this K_d value does not change by changing the concentration of arginine in the range 0.02-0.1 M (see Table I). The enhancement of NADH fluorescence by addition of arginine to the octopine dehydrogenase-NADH complexes permits the evaluation of the dissociation constant of arginine from octopine dehydrogenase-NADH-Arg complexes. The procedure parallels that described several years ago by Theorell and coworkers (see, for instance, Theorell and McKinley-McKee, 1961) in their classical investigations of the fluorescence properties of the ternary complexes of horse liver alcohol dehydrogenase with NADH and isobutyramide. By this technique, $K_{E-R-Arg}$ is found to be 5.0 mM at pH 7.0 phosphate buffer (0.1 M) and room temperature.

The affinity of the reduced coenzyme to the enzyme saturated by octopine is very high: $K_d = 0.9\ \mu M$ at pH 7.0 phosphate buffer and room temperature. The binding of NADH in this ternary complex is tight enough to allow for a direct titration of the enzyme active sites at moderate total concentration of octopine dehydrogenase. Figure 6B reports typical titration experiments.

A linear relationship between the enzyme active site and the steady-state turnover number can be obtained. On this basis, the usual kinetic activity assay of the enzyme can now be directly related to the absolute enzyme active site concentration. A 100% active enzyme has a turnover number of 1000 mol of substrate/mol of enzyme per sec at pH 6.6 and 33°, on the basis of an extinction coefficient of the enzyme of $4.33 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm.

In the case of binary and ternary complexes of NADH, the binding can be measured also by monitoring the enhancement of the coenzyme fluorescence. Figure 6 reports parallel experiments by following the protein fluorescence quenching and the NADH fluorescence enhancement. The two techniques give exactly the same pattern. Analogous agreement is obtained in the measurement of the binding of the binary NADH-octopine dehydrogenase complexes (not reported in the figures).

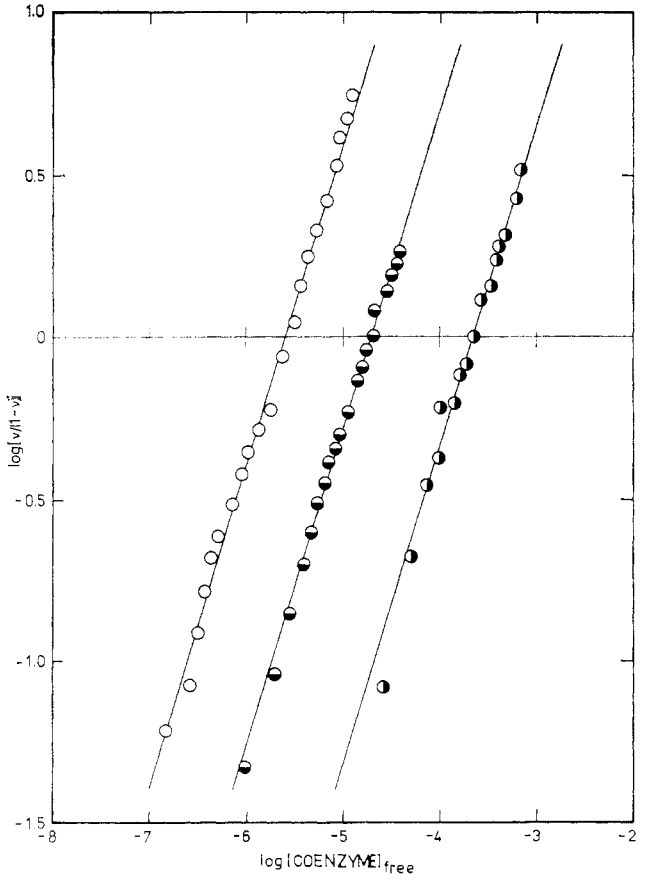


FIGURE 5: Hill plots of NAD^+ and NADH binding to octopine dehydrogenase: (◑) octopine dehydrogenase–NAD^+; (◒) octopine dehydrogenase–NADH; (○) octopine dehydrogenase–NADH–arginine. Conditions were 6.0 μM octopine dehydrogenase for the NAD^+ binding, 4.6 μM octopine dehydrogenase for the NADH binding, 5.0 μM octopine dehydrogenase and 0.02 M arginine for the enzyme–NADH–L-arginine binding. Sodium phosphate buffer (0.1 M, pH 7.0); temperature 20 ± 1°; excitation 290 nm; emission 330 nm. The same set of data for the octopine dehydrogenase–NAD^+ and octopine dehydrogenase–NADH binding is reported in Figure 3; the same set of data for the octopine dehydrogenase–NADH–L-arginine binding is reported in Figure 6A.

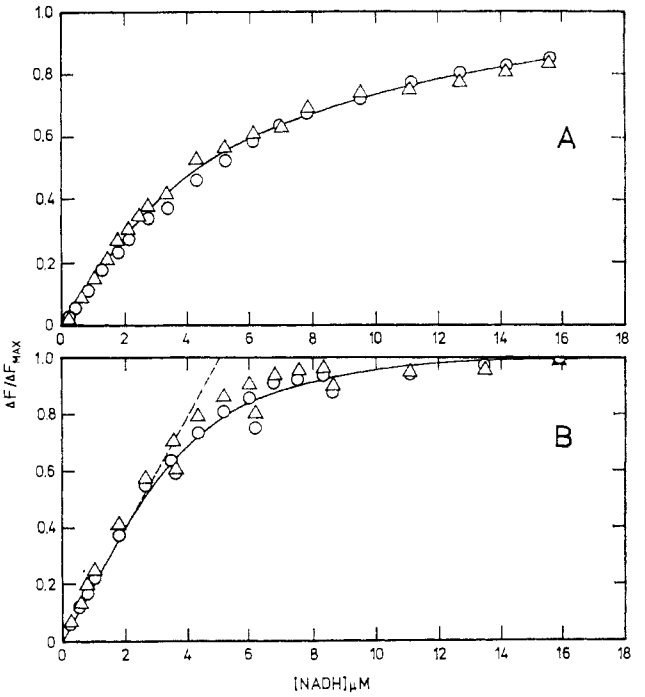


Figure 6: Tryptophan fluorescence quenching (○) and NADH fluorescence enhancement (Δ): (A) in the octopine dehydrogenase–NADH–L-arginine ternary complex; (B) in the octopine dehydrogenase–NADH–octopine ternary complex. The experiments were performed at 20 ± 1° in sodium phosphate buffer (pH 7.0, 0.1 M), in the presence of 0.02 M arginine and octopine, respectively; excitation 290 nm; emission 330 nm. Native enzyme concentration was 5.0 μM. Part B shows a titration of the enzyme active sites by extrapolation of the first portion of the curve.

The quenching of the tryptophan fluorescence upon coenzyme binding provides a fast, routine method to determine the dissociation constants under a large variety of conditions. Table I reports some miscellaneous binding data.

Discussion

The fluorescence properties of octopine dehydrogenase show many similarities to those of oligomeric dehydrogenases, which were already known in literature. The enhancement of NADH quantum yield in binary and ternary complexes of the enzyme, the shift of NADH fluorescence maximum, and the quenching of the tryptophan fluorescence upon coenzyme binding are some of these characteristic properties. The increase of NADH affinity in the enzyme ternary complexes with respect to the coenzyme–enzyme complexes has also been found in several dehydrogenases (Dickinson, 1970; Theorell and Yonetani, 1963). It is interesting to remark in this regard that octopine, which is the product from the side of NADH oxidation, has a more pronounced effect on NADH binding than arginine, which is one of the two substrates, that pyruvate does not have any effect either on NAD^+ or NADH binding, and that arginine has no effect on NAD^+ binding.

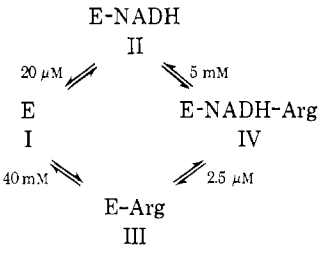
A structural characteristic which seems to be common to octopine dehydrogenase and most dehydrogenases is the hydrophobicity of the binding site. Many independent pieces of evidence pointing to this feature have in fact been accumulated by applying spectroscopic techniques, for instance, to horse liver alcohol dehydrogenase (Brand *et al.*, 1967), to L-α-glycerophosphate dehydrogenase (Kim and Anderson, 1969), to gluconate-6-phosphate dehydrogenases (Rippa and Pontremoli, 1969; Rippa and Picco, 1970), to octopine dehydrogenase (Pho *et al.*, 1970), and to yeast alcohol dehydrogenase (Anderson and Anderson, 1964). In the case of octopine dehydrogenase, the enhancement of NADH quantum efficiency and the blue shift of the coenzyme fluorescence maximum are qualitative but sound confirmations to the same feature.

Another interesting point concerns the mechanism of tryptophan fluorescence quenching. Whereas the quenching produced by NADH can be interpreted in terms of energy-transfer mechanism, the quenching produced by NAD^+ has to be explained in some other way. Notice that no band above 300 nm has been thus far observed in the difference absorption spectra apoenzyme *vs.* enzyme–NAD^+ complex (Pho *et al.*, 1970), and therefore the quenching by NAD^+ cannot occur through excitation energy transfer (as seems to be the case in glyceraldehyde-3-phosphate dehydrogenase). We already discussed the problem of the mechanism of the protein fluorescence quenching by NAD^+ in the case of horse liver alcohol dehydrogenase in a previous paper (Luisi and Favilla, 1970). A direct interaction between NAD^+ and tryptophan could be the most straightforward reason for the protein quenching. Indication of the perturbation of tryptophan(s) upon coenzyme binding in octopine dehydrogenase has been actually

obtained through differential uv spectroscopy (Pho *et al.*, 1970) and spectropolarography (Oriol and Olomucki, 1972). The direct dependence of the protein fluorescence intensity on enzyme activity might also be taken as an indication of the importance of tryptophan residues for having a biologically active enzyme. The hydrophobicity around the emitting tryptophans (fluorescence maximum at 330 nm) is also consistent with the hydrophobicity of the binding site previously discussed. However, none of these indications can be considered conclusive evidence as to the direct interaction of this amino acid residue with the binding coenzyme.

Another question is the comparison between the fluorescence quenching data and those obtained by following the enhancement of NADH fluorescence. Theorell and Tatemoto (1971) and, independently, Holbrook and coworkers (1972) pointed out that in horse liver alcohol dehydrogenase and in some complexes of lactic dehydrogenase the protein fluorescence quenching is not linear with NADH concentration, contrary to the enhancement of NADH fluorescence. Holbrook and others presented a theoretical treatment of this phenomenon (1972) in which the nonlinearity is shown to be due to an intramolecular energy-transfer mechanism between different sites within the same protein molecule. A condition for this nonlinearity is the existence of more than one binding site in the protein. If the protein is a monomer one therefore expects a linear relation between protein fluorescence quenching and coenzyme binding. The linearity we find for octopine dehydrogenase is therefore in agreement with the Holbrook hypothesis.

The values of the dissociation constants obtained for binary and ternary complexes of ODH permit one to draw some mechanistic implications; *e.g.*, consider the following scheme.



The dissociation constant for the step I $\rightleftharpoons$ II can be calculated on the basis of the other three parameters, which have been directly evaluated in this paper. Its value (40 mM) indicates that the affinity of arginine in the binary complexes with the enzyme is one order of magnitude lower than in the ternary complexes of the enzyme containing NADH. An analogous consideration holds for the affinity of coenzyme to binary and ternary complexes of the enzyme (compare the dissociation constants for the steps II $\rightarrow$ I and IV $\rightarrow$ III). Consider also that K_d for the IV $\rightarrow$ III step becomes constant beyond a given arginine concentration (see Table I). All this can be taken as an indication that from the ternary complex IV we have a preferential release of arginine, and that the pathway IV $\rightarrow$ II $\rightarrow$ I is the more likely pathway for the enzyme mechanism. Furthermore, the fact that pyruvate has no effect on the dissociation constant of NADH may suggest that pyruvate may bind only to the species IV, and that it dissociates first from the quaternary (E–NADH–Arg–Pyr) complex.

Investigation of the dissociation constants of suitable substrate analogs in binary and ternary complexes of the enzyme, as well as the direct determination of on and off velocity of substrates and coenzymes, are however needed in order to provide final information on the mechanism of the enzyme.

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Isolation and Purification of Three Egg-Membrane Lysins from Sperm of the Marine Invertebrate *Megathura crenulata* (Giant Keyhole Limpet)†

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ABSTRACT: Three enzymes which lyse the egg-membrane of the marine invertebrate *Megathura crenulata* were obtained from the sperm of a single individual as well as from pooled samples of sperm from several males of the same species. The three enzymes ("lysins") were purified to homogeneity by a combination of gel filtration and ion-exchange chromatography. The three egg-membrane lysins were different as judged by chromatographic behavior, gel electrophoresis in the presence of sodium dodecyl sulfate, amino acid composition, isoelectric point, and circular dichroism spectra. The three egg-membrane lysins had molecular weights of 57,250 (±572), 53,688 (±1387), and 58,000 (±580) as determined by sedimentation equilibrium in the presence of 6 M guanidine–HCl. Similar experiments carried out in the absence of guanidine–HCl yielded similar molecular weight values, showing that all three enzymes exist as monomeric species.

Egg-membrane lysins are enzymes, found in the sperm extracts of marine invertebrates and other animals, that are capable of dissolving the egg vitelline membrane[1] (Dan, 1967). Tyler (1939) was able to show the existence of an egg-membrane lysin in the sperm extract of the giant keyhole limpet *Megathura crenulata* and suggested that such an enzyme might facilitate the penetration of the spermatozoon through the vitelline membrane into the egg. Further studies on the *Megathura crenulata* egg-membrane lysin were done by Krauss (1950). Recently, an egg-membrane lysin having a molecular weight of 8800 has been isolated from a sperm extract of *Tegula pfeifferi* (Haino, 1971). A similar enzyme, reported to be located in the acrosomes of mammalian spermatozoa, has been isolated (Stambaugh and Buckley, 1972; Zaneveld *et al.*, 1972) and it is thought that this enzyme dissolves a small tunnel in the zona pellucida through which the spermatozoon can penetrate the egg (Stambaugh and Buckley, 1972).

Further knowledge of such lysins could be helpful in understanding the fundamentals of fertilization. Egg-membrane lysins may serve as tools in elucidating the structure and function of the vitelline membrane, in understanding species specificity, enhancing fertilization, or in developing fertilization inhibitors. This study describes the isolation, purification, and some properties of three egg-membrane lysins from sperm extracts of *Megathura crenulata*.

Experimental Section

Materials. Giant keyhole limpets (*Megathura crenulata*) were purchased from Pacific Bio-Marine, Venice, Calif. Sepharose 6-B, Sephadex G-75, and Sephadex QAE-50 were purchased from Pharmacia; cellulose phosphate was obtained from Sigma; hydroxylapatite HTP and Bio-Gel P-150 were purchased from Bio-Rad Laboratories.

Methods. Isolation of Sperm. Testes of dissected limpets were suspended in 0.01 M Tris-HCl (pH 8.3) containing 0.5 M NaCl allowing the sperm to shed into the buffer solution. The mixture was passed through cheesecloth and the sperm suspension that passed through was centrifuged at 5000 rpm in a Sorvell-SS 34 rotor for 10 min. The supernatant was discarded and the pellet containing the sperm was washed twice with the Tris buffer.

Isolation of Egg-Membranes. Ovaries of dissected limpets were suspended in 0.005 M glycine–NaOH (pH 8.55) containing 0.5 M NaCl and stirred gently with a magnetic stirrer at 4° overnight. The suspension was then passed through cheesecloth and centrifuged at 9000 rpm in a Sorvall GS-3 rotor for 40 min. The pellet, containing the jellyless eggs, was resuspended in the glycine–NaOH buffer. The eggs were then homogenized in a Teflon homogenizer (Thomas, Phil.) causing the egg cytoplasm to escape from the sack-like membrane. The homogenization was followed by inspecting samples under the light microscope. The suspension was then passed through a nylon screen cloth, Nitex 30 (Kressilk Products, Inc.); all the cell debris except the egg-membrane passed through. After thorough washing with 0.005 M glycine–NaOH containing 0.5 M NaCl, the membranes were collected from the screen and stored in the glycine–NaOH buffer at 4°. The membranes seemed homogeneous as judged by light

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[1] The term egg-membrane does not imply any definite known structure such as a lipid bilayer. Other names which were used to describe the same structure are vitelline membrane, vitelline coat, or vitelline envelope. Since the actual molecular structure of this morphological entity is not known at present, we prefer to use the name which has been in longest use, namely egg-membrane.